Effects of Using Chitosan (CHS) Scaffolds on Bone Healing in Rat Animal Model: Radiological and Histopathological Evaluations

Abstract

Introduction: Bone fracture is one of the most common bone injuries in the world. It is, therefore, not surprising that bone healing is a critical orthopedic problem. Currently, bone grafts (autografts, allografts and xenografts) have been used as bone replacement strategies. The purpose of this study was to evaluate the efficacy of application of chitosan (CHS) as an osteo-inducer in the process of bone healing.

Materials & Methods: Fifteen male rats, in the same age and weight range, were divided into three groups of 5. 3 mm of metaphysis of the radius bone of each animal was removed. The defect was left empty in the first , filled with autograft in the second ,and filled with CHS in the third group. Radiographs of the defects were obtained on 28th and 56th day post-operation. The animals were euthanized according to human ethics and the defects were investigated histologically on the 56th post-operation day.

Results & Discussion: According to radiographic and histopathologic results, CHS indicated a improved effect ,and improved bone healing process in comparison with the other two treatment modalities.

Conclusion: It can be concluded that use of chitosan improves the potential for bone formation and bone healing criteria. These composite materials, however, deserve more studies.

Keywords: Chitosan, Bone regeneration, Bone grafting, Rat.

Accepted: 32 days before printing

Fatemeh Heidari, DVM[1], Amin Bigham-Sadegh, PhD[1], Ahmad Oryan, PhD[2], Abdolhamid Meimandi-Parizi, PhD[1], Alireza Shaikhzadeh, DVM[1], Seyed Ali Afzali, DVM[1], Maryam Askari, DVM[1]

1. Department of Surgery and Radiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
2. Department of Pathology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

Corresponding Author:
Amin Bigham-Sadegh, PhD
Email address:
dr.bigham@gmail.com

Introduction

Bone is a combined structure composed of both mineral and organic components. The primary mineral component is calcium phosphate, whereas the main organic component consists of collagen[(1)]. Bones are not just lifeless scaffolding, but are dynamic, supportive , protective and hematopoiesis organs. The difference of bone from another connective tissues are hardness and strength. These specific capabilities are due to the incorporation of mineral salts into a collagen fibers matrix, non-collagenous proteins, and other components[(2)].

A critical-sized defect in bone is a lost or resected part of bone because of trauma, osteomyelitis, tumor, or other reasons , that cannot heal on its own[(3)]. The most common cause of bone defects is fracture and the healing process is a combination of biological and mechanical aspects[(4)]. The appropriate management of bone fractures and bone defects still remains unresolved and 5–10% of them have failure in healing ,resulting in nonunion[(5,6)]. Chitosan is a natural polysaccharide widely used in medicine, derived from chitin. It is also found as a component of fungal cell walls, the exoskeletons of crustaceans and insects, and fish scales[(7,8)]. It is inexpensive, adaptable, available, nontoxic, biocompatible, and has a numerous natural source also it is antimicrobial, antioxidant, anti-inflammatory, and anticancer characteristics , with excellent hemocompatibility, and hemostatic activity—chitosan has attracted significant attention[(7)]. Chitosan, resembling glycosaminoglycans, is a natural component of the extracellular matrix that provides a microenvironment for growing cell and supporting the proliferation, differentiation, and mineralization of osteoblasts[(8)].

This study conducted to assess the effect of chitosan powder on healing of bone in rats, through radiological and histopathological analyses. By quantitatively and qualitatively examining the healing and union process, this study seeks to highlight the advantages and limitations of this method, with the ultimate goal of introducing scaffolds of sufficient strength for the treatment of such fractures in the future.

Materials & Methods

This study was conducted in May 2024 at the Faculty of Veterinary Medicine, Shiraz University, in the Department of Surgery and Radiology. Chitosan was available in commercial powder form.

Animals and Grouping

A total of 15 adult male rats, weighing 200 ± 20 g, were housed in a cage for one month to acclimatize to the environment and to undergo complete health assessment. The rats ,randomly were divided into three groups: untreated (Sham), autograft, and chitosan powder–treated groups.

Surgical Procedure

Anesthesia was induced with 0.1 mL of 10% ketamine combined with 0.1 mL of midazolam. The surgical site was aseptically prepared. An incision, twice the width of the defect, was made on the skin along the left forelimb over the radius, exposing the bone. Using an electric file and abrasive tool, a 3-mm segment of the radial metaphysis along with the surrounding periosteum was removed. The size of the defect was defined as twice the diameter of the bone. To prevent thermal necrosis, bone cutting was performed under continuous irrigation with normal saline. Any intraoperative blood clots were carefully removed from the defect to prevent them from serving as a scaffold for new bone formation.

In the untreated group, the defect site was left empty, in the second group, the autologous bone graft was used for filling the defect , and in the third group, it was filled with chitosan powder. After completion, the surgical incision was sutured.

Postoperative Care and Evaluation

The rats were monitored daily for forelimb use, weight-bearing on the operated limb, general condition, appetite, and physical activity. Local wounds, inflammation, swelling, stiffness, redness, hemorrhage, defect filling, and lack of bone healing were also assessed. Radiographs were taken over an eight-week period post-surgery to evaluate bone healing radiologically. Lateral radiographs of the forelimb were obtained on days 0, 28, and 56. Evaluation and scoring of the obtained radiographs with a modified Lane and Sandhu grading system was done.(Table 1)

Table 1. Modified Lane and Sandhu Radiographic Grading System

Bone Formation	Grading
No evidence of bone formation	0
Bone formation with 25% defect filling	1
Bone formation with 50% defect filling	2
Bone formation with 75% defect filling	3
Bone formation with complete (100%) defect filling	4
Union (Proximal and Distal)	Grading
No union	0
Probable union	1
Complete union	2
Remodeling	Grading
No evidence of remodeling	0
Mild evidence of remodeling	1
Complete remodeling	2

Histopathological Evaluation

After eighth week, the rats were humanely sacrificed, and tissue sampling was performed for histopathological assessment. The isolated tissue samples were immediately fixed in freshly prepared 10% formalin for three days. After 24 hours, the formalin solution was replaced. Subsequently, the samples were decalcified and the samples were regularly examined with a fine needle to confirm the removal of mineral components. Complete decalcification was confirmed when the needle could penetrate the sample without resistance. To remove residual acid, the samples were rinsed under running water for 30 minutes[9].

The samples were then embedded in paraffin blocks, sectioned at 5 µm thickness, then with hematoxylin and eosin was stained for histopathological evaluation. Bone healing was assessed using the Emery scoring system[10] (Table 2). In addition, the number of osteoblasts, osteocytes, osteoclasts, and trabeculae numbers was histologically examined across four fields under a light microscope at 100× magnification. All evaluations of the lesion site were

done by an experienced pathologist - blinded to the group allocation to avoid bias.

Table 2: Bone healing was assessed using the Emery scoring system

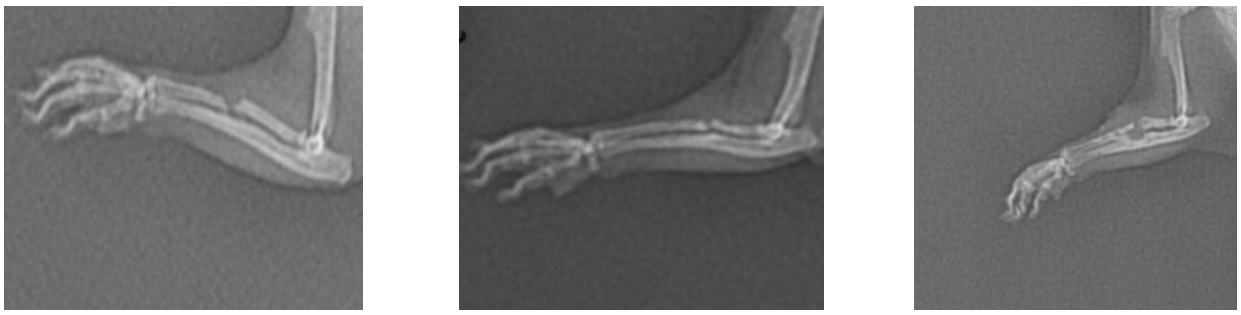
Defect Condition	Grade
Empty	0
Fibrous connective tissue only	1
Predominantly fibrous tissue over cartilage	2
Predominantly cartilage over fibrous tissue	3
Cartilage only	4
Predominantly cartilage over bone	5
Predominantly bone over cartilage	6
Bone only	7

Statistical Analysis

The obtained results were first analyzed using the Kruskal–Wallis H test (ANOVA). When the P-value was less than 0.05, further analysis was performed using the Mann–Whitney U test. In this test, values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS software.

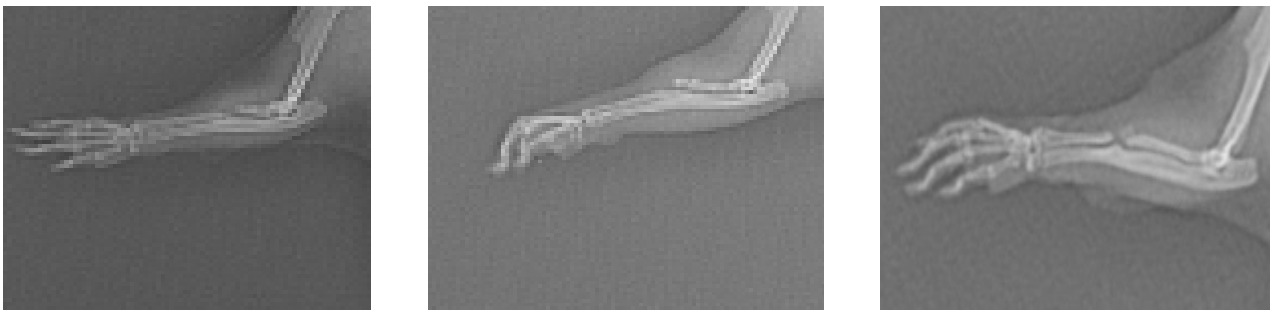
Results

None of the rats died during the study period. All animals maintained normal appetite and body weight. Radiographs of the forelimbs were taken on days 0, 28, and 56 in lateral view. Each radiograph was scored based on the radiographic signs observed, and the total score was calculated (Figure 1 and 2). Qualitative data were reported as median (minimum–maximum). Statistical comparisons of the data were performed using the Kruskal–Wallis H test and the Mann–Whitney U test (Table 3).



(a) (b) (c)

Figure 1. Radiological views of the groups on day 28: untreated group (a), autograft group (b), and chitosan-treated group (c).



(a) (b) (c)

Figure 2: Radiological views of the groups on day 56: untreated group (a), autograft group (b), and chitosan-treated group (c).

Table 3: Comparison of Radiological Evaluation of Groups at Weeks 4 and 8

Day / Group	Untreated Median (min–max)	Autograft Median (min–max)	Chitosan Median (min–max)	P-value
Day 28	0 (0–2)*	4 (4–6)*	2 (2–3)	0.001
Day 56	3 (2–4)*	6 (4–10)*	3 (2–5)	0.02

*= statistically significant differences among groups.

Statistical analysis showed significant differences between the untreated and the autograft group (P = 0.008), also between the untreated and the chitosan group (P = 0.03).

These findings indicate that the untreated group performed significantly worse than both treated groups. In addition, there was a statistically significant difference between the autograft and chitosan groups (P = 0.008), demonstrating that the autograft group showed superior performance. Statistical analysis also confirmed a significant difference between the untreated and autograft groups (P = 0.008), supporting the conclusion that the autograft group outperformed the untreated group. Finally, a significant difference was shown between the autograft and chitosan groups (P = 0.01), indicating that the autograft group had better outcomes.

Histopathological Evaluation

In the untreated group, disorganized soft connective tissue was observed within the defect site, with blood vessels, small amounts of cartilage, and limited bone formation at the edges of the fracture site (Figure 3).

In the autograft group, the defect site contained a substantial amount of cartilage and bone. Bone formation appeared to occur predominantly via intramembranous ossification, with most connective tissue directly transforming into bone.

Considerable amounts of cartilage and soft connective tissue were also noted at the defect site (Figure 4).

In the chitosan-treated group, histopathological examination revealed limited amounts of bone, soft connective tissue, and cartilage formation within the defect area (Figure 5).

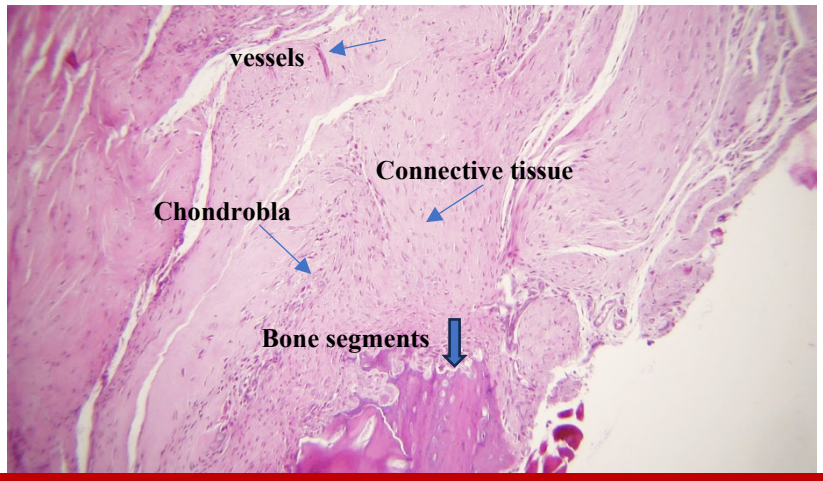


Figure 3: Histopathology of the defect in the untreated group (100×, H & E).

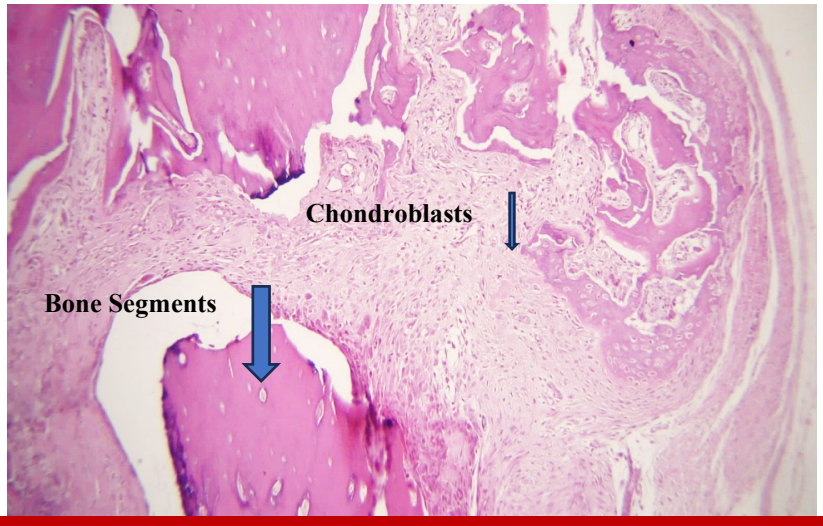


Figure 4: Histopathology of the defect in the autograft group (100×, H & E).

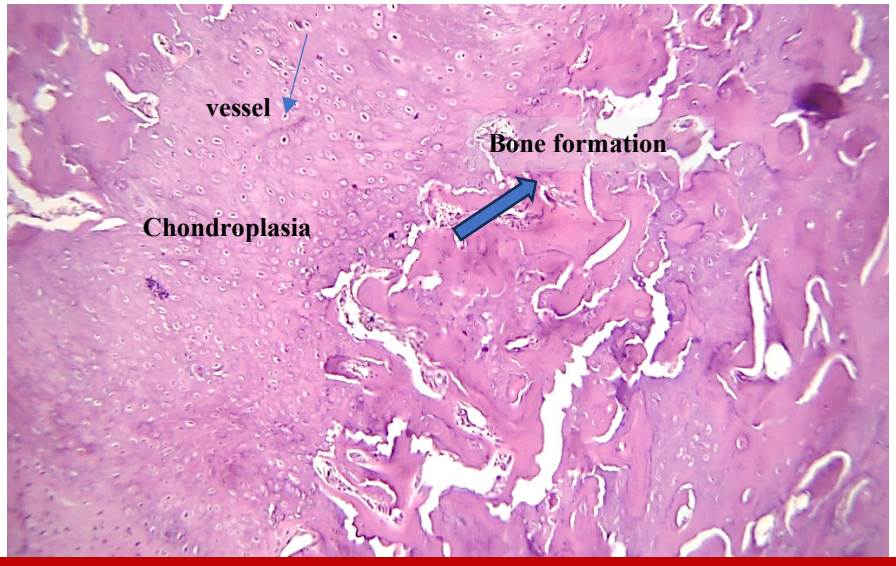


Figure 5: Histopathology of the defect in the chitosan-treated group (100×, H & E).

Discussion

Today, identifying an ideal biomaterial for managing the large defect of bone , delaying union, and nonunion remains a major issue for research in the worldwide. Numerous studies about the bone regeneration, have reported good results[11]. The present study investigated the radiological and histopathological effects of chitosan powder on bone healing in a rat model. The study was designed on the radius because it is the only bone in which a full-thickness defect can be created without the use of pins, screws, or plates, due to its connection with the ulna at the distal end.

Selecting an appropriate animal model and simulate fractures carefully, can show healing processes of normal and abnormal bone, and facilitate the development of novel therapeutic approaches[12]. Rodents and rabbits are frequently chosen due to favorable economic factors. In this study, the animal model were rats, and the radius served as the fracture model.

An important aspect of this study was the inclusion of an autograft treatment group, regarded as the “gold standard,” against which all other groups were compared. In medical and social literatures, the term “gold standard” refers to the best available method or reference against which other interventions are evaluated[13]. Three groups were used in this study: untreated, autograft, and chitosan-treated. The study period was 56 days, and the results demonstrated a positive effect of chitosan on bone repair. Radiological evaluations were performed at weeks 4 and 8, while histopathological assessments were conducted at the end of week 8.

Radiographic imaging was important in current clinical diagnosis and also for monitoring the progression of disease, and therapeutic follow-up. Techniques such as radiography, CT scan , MRI, ultrasound, and nuclear imaging studies are essential tools in diagnostic in bone problems[14]. Histological staining followed by histomorphometry provides another method to evaluate fracture callus, allowing differentiation between tissue types (cartilage, soft connective tissue, degree of ossification, cellular components, bone marrow). This involves staining thin tissue sections with selective dyes, enabling both visual identification and computer-assisted analysis[15]. Differentiating cartilage from soft connective tissue is particularly important, as connective tissue filling a fracture defect may indicate poor or absent bone formation, whereas cartilage and newly formed bone indicate active healing. Excess soft connective tissue is a key marker of nonunion[16].

Histopathological analysis in the present study revealed that the chitosan-treated group performed more effectively than the untreated group. The presence of cartilage and bone in the chitosan group indicates its positive influence on bone repair, facilitating secondary and endochondral bone formation. Chitosan promotes healing through enhanced cell proliferation, angiogenesis, and modulation of inflammatory responses[17].

In the autograft group, soft connective tissue was observed, which appeared to transform directly into bone, with minimal cartilage at the defect site. Bone formation in this group occurred mainly via intramembranous ossification, indicating primary and direct bone healing[18]. In the untreated group, disorganized bone formation and limited vascularization were observed, suggesting reduced angiogenesis during repair. Overall, the untreated group showed the weakest bone healing among all groups.

This study also demonstrated that chitosan enhanced angiogenesis compared with the untreated group, which had empty defects. Increased vascularization improves the bone's potential for repair[19]. Chitosan that was used in vitro study can enhance adhesion and proliferation of osteoblasts and mesenchymal stem cells[20].

Hu et al. evaluated the effectiveness and mechanism of nanofiber scaffolds of chitosan on healing of bone . Critical-sized defects in the femur of rats was treated with chitosan nanofiber scaffolds, and the opposite femur used as control. Imaging analyses demonstrated that the scaffolds significantly improved bone repair, increasing trabecular bone thickness while reducing trabecular separation[21]. Our study confirms that these effects are enhanced with chitosan-based treatments.

Arian et al. reported of a composite scaffold consisting of chitosan (CS), gelatin (Gel), and platelet gel (PG), named CS-Gel-PG, on critical-sized in radial defects of both sides in 40 rats. The autograft and CS-Gel-PG groups had better formation of new bone, bone and cartilage tissue density, bone volume, and mechanical performance compared with untreated, CS, and Gel-PG groups[11].

Chitosan scaffolds represent a promising option for healing of a critical-sized defect on radial bone; But the mechanisms underlying enhanced bone healing by these natural materials require further investigation. Future research should focus on optimizing antibacterial and osteogenic effects of chitosan in physiological conditions and improving its biological performance. Such evidence may overcome current challenges and offer new perspectives for treating infected bone defects[22].

Conclusion

The results of this study indicate that chitosan enhances regeneration of bone with mechanical stimulation of bone tissue. Chitosan exerts its effects by promoting proliferation of osteoblasts and mesenchymal stem cells, and by supporting osteogenic differentiation of adipose-derived stem cells. Overall, these findings suggest that tissue engineering and biomaterials can serve as viable alternatives to bone grafts. Selecting a cost-effective and readily available biomaterial may provide an alternative to autografts and allografts, positioning tissue engineering as a promising approach for treatment of bone defects in the future.

Acknowledgment

The authors sincerely thank Dr. Alireza Sheikhzadeh for his invaluable assistance in the successful completion of this study.

References

1 Indurkar A, Choudhary R, Rubenis K, Locs J. Role of carboxylic organic molecules in interfibrillar collagen mineralization. Front Bioeng Biotechnol. 2023;11:1150037. DOI: 10.3389/fbioe.2023.1150037.
2 Šromová V, Sobola D, Kaspar P. A Brief Review of Bone Cell Function and Importance. Cells. 2023; 12(21):2576. doi: 10.3390/cells12212576.
3 Huang E.E, Zhang N, Ganio E.A, Shen H, Li X, Ueno M, et al. Differential dynamics of bone graft transplantation and mesenchymal stem cell therapy during bone defect healing in a murine critical size defect. J Orthop Translat. 2022;36:64-74. DOI: 10.1016/j.jot.2022.05.010.
4 Ghiasi M.S, Chen J, Vaziri A, Rodriguez E.K, Nazarian A. Bone fracture healing in mechanobiological modeling: A review of principles and methods. Bone Rep. 2017;6:87-100. DOI: 10.1016/j.bonr.2017.03.002
5 Toosi S, Behravan N, Behravan J. Nonunion fractures, mesenchymal stem cells and bone tissue engineering. J Biomed Mater Res A. 2018;106(9):2552-62. DOI: 10.1002/jbm.a.36433.
6 Einhorn T.A, Gerstenfeld L.C. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol. 2015;11(1):45-54. DOI: 10.1038/nrrheum.2014.164.
7 Ul-Islam M, Alabbosh K.F, Manan S, Khan S, Ahmad F, Ullah M.W. Chitosan-based nanostructured biomaterials: Synthesis, properties, and biomedical applications. Adv Ind Eng Poly Res. 2024;7(1):79-99.
8 Pattnaik S, Nethala S, Tripathi A, Saravanan S, Moorthi A, Selvamurugan N. Chitosan scaffolds containing silicon dioxide and zirconia nano particles for bone tissue engineering. Int J Biol Macromol. 2011;49(5):1167-72. DOI: 10.1016/j.ijbiomac.2011.09.016.
9 Ibrahim L.M, Jassim R.K,, Al Gabban A. Biomechanical and histomorphometric analysis of max phase (Ti2AlC) alloy as an implant. J Res Med Dent Sci. 2022;10(8):51–7.
10 Fadhil E, Dosh R.H, Wally Z.J, Haider J. Histological evaluation of the effects of bone morphogenetic protein 9 and angiopoietin 1 on bone healing. J Taibah Univ Med Sci. 2023;18(5):954-63. DOI: 10.1016/j.jtumed.2023.02.005.

11 Oryan A, Bigham-Sadegh A, Abbasi-Teshnizi F. Effects of osteogenic medium on healing of the experimental critical bone defect in a rabbit model. Bone. 2014;63:53-60. DOI: 10.1016/j.bone.2014.02.010.
12 Gao H, Huang J, Wei Q, He C. Advances in Animal Models for Studying Bone Fracture Healing. Bioengineering (Basel). 2023;10(2):201. DOI: 10.3390/bioengineering10020201.
13 Brodsky S.L, Lichtenstein B. The Gold Standard and the Pyrite Principle: Toward a Supplemental Frame of Reference. Front Psychol. 2020;11:562. DOI: 10.3389/fpsyg.2020.00562.
14 Math K.R, Berkowitz J.L, Paget S.A, Endo Y. Imaging of Musculoskeletal Infection. Rheum Dis Clin North Am. 2016;42(4):769-84. DOI: 10.1016/j.rdc.2016.07.010.
15 Knox A.M, McGuire A.C, Natoli R.M, Kacena M.A, Collier C.D. Methodology, selection, and integration of fracture healing assessments in mice. J Orthop Res. 2021;39(11):2295-309. DOI: 10.1002/jor.25172.
16 Han Z, Bhavsar M, Leppik L, Oliveira K.M.C, Barker J.H. Histological Scoring Method to Assess Bone Healing in Critical Size Bone Defect Models. Tissue Eng Part C Methods. 2018;24(5):272-9. DOI: 10.1089/ten.TEC.2017.0497.
17 Mawazi SM, Kumar M, Ahmad N, Ge Y, Mahmood S. Recent Applications of Chitosan and Its Derivatives in Antibacterial, Anticancer, Wound Healing, and Tissue Engineering Fields. Polymers (Basel). 2024;16(10).
18 Ferraz MP. Bone Grafts in Dental Medicine: An Overview of Autografts, Allografts and Synthetic Materials. Materials (Basel). 2023;16(11):1351. DOI: 10.3390/polym16101351.
19 Saran U, Gemini Piperni S, Chatterjee S. Role of angiogenesis in bone repair. Arch Biochem Biophys. 2014;561:109-17. DOI: 10.1016/j.abb.2014.07.006.
20 Zhao X, Li P, Guo B, Ma P.X. Antibacterial and conductive injectable hydrogels based on quaternized chitosan-graft-polyaniline/oxidized dextran for tissue engineering. Acta Biomater. 2015;26:236-48. DOI: 10.1016/j.actbio.2015.08.006.
21 Ho M.H, Yao C.J, Liao M.H, Lin P.I, Liu S.H, Chen R.M. Chitosan nanofiber scaffold improves bone healing via stimulating trabecular bone production due to upregulation of the Runx2/osteocalcin/alkaline phosphatase signaling pathway. Int J Nanomedicine. 2015;10:5941-54. DOI: 10.2147/IJN.S90669.
22 Tian Y, Wu D, Wu D, Cui Y, Ren G, Wang Y, et al. Chitosan-Based Biomaterial Scaffolds for the Repair of Infected Bone Defects. Front Bioeng Biotechnol. 2022;10:899760. DOI: 10.3389/fbioe.2022.899760.